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THE EFFECT OF EXTRACELLULAR CALCIUM ON THE CONTRACTILE ACTION OF ENDOTHELIN

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The tension developed by rat aortic strips in response to endothelin-1 is determined by three types of mechanisms: a $[Ca^{2+}]o$ independent mechanism, L-type Ca^{2+} channels and a $[Ca^{2+}]o$ dependent, verapamil insensitive, mechanism. Their relative contributions to the tension recorded 30 minutes after the addition of 50 nM endothelin-1 were 43%, 34% and 23%. Upon longer exposures to endothelin-1, the whole tension could be abolished by reducing $[Ca^{2+}]o$ to 20 nM. Endothelin-1 induced contractions were highly sensitive to changes in free $[Ca^{2+}]o$. The EC50 value for the $[Ca^{2+}]o$ dependence of endothelin-1 induced contractions was 600 nM, a value 400 times lower than the corresponding value found for KCl induced contractions (250 μM). These results suggest that extracellular Ca^{2+} is necessary for full tension development in response to endothelin-1 but that a major action of endothelin-1 is to increase the sensitivity of pharmacomechanical coupling mechanisms to Ca^{2+}.

Endothelin-1 is a potent vasoconstricting peptide that is synthesized by the vascular endothelium (1). Different mechanisms have been proposed to mediate its contractile action: (i) activation, either direct or indirect, of L-type Ca^{2+} channels (2,3), (ii) activation of phospholipase C with the resulting inositol trisphosphate mediated intracellular Ca^{2+} mobilization (3-6) and diacylglycerol mediated activation of protein kinase C (6-8) and (iii) opening of a non selective cationic channel that is permeable to Ca^{2+} (3). All these mechanisms are expected to lead to contractions but their relative contributions to the tension observed have not yet been determined. In this paper we analyze the effect of extracellular $[Ca^{2+}]$ on the contractile action of Et-1 using isolated aortic strips. We show that a previously unrecognized action of Et-1 is to increase the sensitivity of aortic strips to Ca^{2+}.

MATERIAL AND METHODS

Thoracic aortae were isolated from 6-8 week old female Wistar Kyoto rats, cut into 4 mm strips and the intima denuded. Arterial segments were equilibrated at 37°C for

Abbreviations: Et-1, endothelin-1; EGTA, ethyleneglycol-bis (β-aminoethyl ether) N,N,N',N' tetracaetic acid.

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1 hour under 2 g resting tension in a Krebs-Ringer solution (120 mM NaCl, 4.8 mM KCl, 1.2 mM $CaCl_2$, 1.3 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 5.8 mM glucose) gassed with 95%O_2, 5% CO_2. After equilibration, each strip was conditionned by three contraction relaxation cycles with 40 mM KCl and then used for the experiments. The increase in isometric tension was recorded using a Gould-Brush 2600 polygraph. For determining the $[Ca^{2+}]o$ dependence of Et-1 induced tension, aortic strips, incubated in a 1.2 mM $CaCl_2$, 4 mM EGTA solution (free $[Ca^{2+}]o$ = 20 nM), were precontracted with 50 nM Et-1. After 30 minutes, once maximum tension had been reached, small amounts of $CaCl_2$ were added to the bath solution to increase free $[Ca^{2+}]o$ and tension was recorded. Free Ca^{2+} concentration was computed as described previously (9). Synthetic Et-1 was purchased from Penninsula. Phorbol myristate acetate was from Sigma. Means ± SE are indicated.

RESULTS AND DISCUSSION

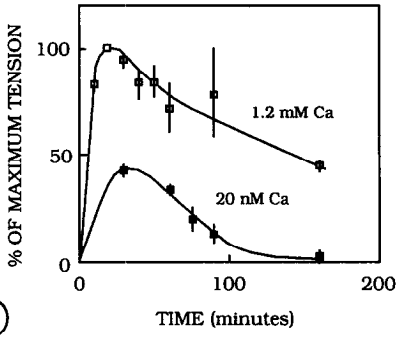
Cytosolic free Ca^{2+} is thought to be the intracellular second messenger that, through the phosphorylation of myosin light chains, permits the activation of myosin ATPase by actin (10). Ca^{2+} may originate either from intracellular stores or from extracellular spaces *via* the opening of membrane Ca^{2+} channels. The role of Ca^{2+} uptake pathways in the contractile action of Et-1 was analyzed using (±)verapamil, an inhibitor of L-type Ca^{2+} channels and low $[Ca^{2+}]o$ conditions. The ratio of the tension developed by aortic rings in response to 50 nM Et-1 to that produced during a prepulse with 40 mM KCl was 1.80 ± 0.11 (n = 19). This value was 1.18 ± 0.13 (n = 4) in the presence of 50 μM (±)verapamil and 0.78 ± 0.07 (n = 10) when free $[Ca^{2+}]o$ was 20 nM. These values indicate that three mechanisms contributed to tension: a $[Ca^{2+}]o$ independent mechanism, L-type Ca^{2+} channels and a verapamil insensitive, $[Ca^{2+}]o$ dependent, mechanism. Their relative contributions to the tension measured 30 minutes after the addition of 50 nM Et-1 were 43%, 34% and 23%. These data show that L-type Ca^{2+} channels, that are activated by the membrane depolarization induced by Et-1 (3), contribute only to part of the contractile action of Et-1 in the rat aorta. They also indicate that a Ca^{2+} uptake pathway that was not L-type Ca^{2+} channel contributed to tension. This pathway could be the non

Figure 1: The contribution of external Ca^{2+} to Et-1 induced tension. Representative tracing showing the influence of changes in free $[Ca^{2+}]o$ on the tension developped in response to 50 nM Et-1. An aortic ring bathed into a 1.2 mM $CaCl_2$, 4 mM EGTA solution (free $[Ca^{2+}]o$ = 20 nM) was first treated with Et-1. Once tension had reached a maximum, $[Ca^{2+}]o$ was increased to 1.2 mM by washing off EGTA and decreased back to 20 nM by the addition of 4 mM EGTA. This sequence of operations was repeated three times on the same preparation. The arrow indicates the time of Et-1 addition. In the upper tracing, the open bars represent periods in which free $[Ca^{2+}]o$ was 20 nM, black bars represent periods in which $[Ca^{2+}]o$ was 1.2 mM.

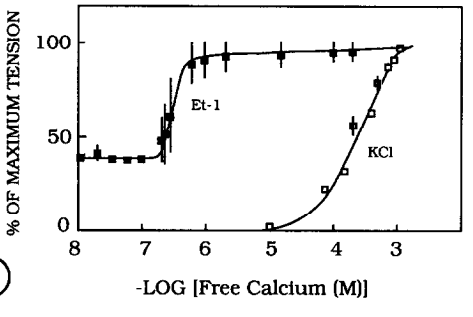
selective, Ca^{2+} permeable, channel that has previously been shown to be activated by Et-1 in aortic myocytes and that is responsible for its depolarizing action (3). Its contribution to total tension is however less than that of L-type Ca^{2+} channels.

Figure 1 shows the results of an experiment in which an aortic strip was precontracted with 50 nM Et-1 in a 20 nM free [Ca^{2+}] solution and then exposed to a high (1.2 mM) Ca^{2+} solution. Changing the extracellular concentration of Ca^{2+} led to rapid and reversible changes in tension. The time required to reach half maximum tension when [Ca^{2+}]o was raised from 20 nM to 1.2 mM was 1.90 ± 0.10 minutes (n = 10). This value is comparable to the half time for tension development in response to 40 mM KCl (1.62 ± 0.06 minutes, n = 17). It was much smaller than the half time for tension development in response to 50 nM Et-1 (5.36 ± 0.14 minutes, n = 15). Figure 1 also shows that low free [Ca^{2+}]o conditions were less potent to reverse Et-1 induced contractions at the onset of tension development than after a prolonged exposure to Et-1. This indicated that the contribution to tension of [Ca^{2+}]o independent mechanisms was not the same at different times of the action of Et-1. Figure 2 summarises the results of several experiments and shows that the importance of the [Ca^{2+}]o independent component of tension was greatest 30 minutes after the addition of Et-1. Then it decreased. After a 2 hour exposure to Et-1, the whole tension could be abolished by decreasing free [Ca^{2+}]o to 20 nM.

The observation that a prolonged, [Ca^{2+}]o independent mechanism maintained tension in Et-1 treated aortic strips was surprising for this type of mechanism is usually assumed to be dependent on inositol trisphosphate mediated intracellular Ca^{2+} release. Et-



(2)



(3)

Figure 2: Time course of tension development by Et-1 treated aortic rings bathed into a 1.2 mM or a 20 nM Ca^{2+} solution. For the low Ca^{2+} experiments, contractions were initiated at 1.2 mM [Ca^{2+}]o. After different times, EGTA (4 mM) was added to reduce free [Ca^{2+}]o to 20 nM and tension was recorded. This procedure prevented the preparation to be exposed to EGTA for very long periods of time. Tensions were expressed relatively to the maximum tension recorded in each experiment. Bars represent means ± SE (n = 3-5).

Figure 3: The extracellular Ca^{2+} dependence of Et-1 and KCl induced contractions. Symbols used are: 50 nM Et-1 induced tension (■) and 40 mM KCl induced tension (□). Experiments were performed as described in Materials and Methods. Means ± SE (n = 2-4) are shown. Tensions were expressed relatively to the tension recorded at the end of each experiment after washing off EGTA and raising [Ca^{2+}]o to 1.2 mM.

1, like most vasoconstrictors, activates phospholipase C and induces the production of inositol trisphosphate from phosphatidylinositol and the release of Ca^{2+} from intracellular stores (3-6). This action is however limited to the first minutes of its action (3-6), suggesting that an additional mechanism is involved. It could be a sensitization of the contractile apparatus to Ca^{2+} (11-14). Figure 3 shows that an increase in free $[Ca^{2+}]o$ from 20 nM to 200 nM did not alter tension of aortic rings that have been precontracted with 50 nM Et-1. However when the free $[Ca^{2+}]o$ was raised above a threshold concentration of 200 nM, tension increased and reached a maximum at 2 μM $[Ca^{2+}]o$. The EC_{50} value for the free $[Ca^{2+}]o$ dependence of Et-1 induced contractions was 600 nM. Figure 3 also shows that at micromolar free external Ca^{2+} concentrations, KCl induced contractions were prevented. KCl acts by depolarizing cells and by opening L-type Ca^{2+} channels. Its action was observed only for $[Ca^{2+}]o > 10$ μM. The EC_{50} value for the free $[Ca^{2+}]o$ dependence of KCl induced contractions was 250 μM, a value 400 times higher than the corresponding value found for Et-1 induced contractions. These results indicate that the contractile action of Et-1 may be associated to a large increase in the sensitivity of the pharmacomechanical coupling mechanisms to Ca^{2+}.

The contractile action of Et-1 is similar in many respects to that of phorbol esters. Both Et-1 and phorbol esters induce slowly developing contractions that are partly dependent on $[Ca^{2+}]o$ and that are associated to an increased sensitivity to Ca^{2+} (15,16). Et-1 inducing a sustained production of diacylglycerol by aortic myocytes (6-8), it could be that protein kinase C is involved in the observed sensitization to Ca^{2+}. We observed however (i) that Et-1 developed much larger tensions that phorbol myristate acetate and (ii) that a 1 hour treatment of aortic strips with 100 nM phorbol myristate acetate did not prevent Et-1 to produce a large and slowly developping tension. These observations indicate that the production of diacylglycerol in response to Et-1 (6-8) and the resulting activation of protein kinase C were unlikely to be the only mechanism by which Et-1 sensitizes cells to Ca^{2+}.

Taken together, these results show that several pharmacomechanical coupling mechanisms operate sequentially to determine tension in Et-1 treated aortic strips and that a sensitization of the cells to Ca^{2+} is probably one of the most important mechanisms by which Et-1 exerts its contractile action. Because of the stability of its contractile action, Et-1 may be a very useful tool to analyze the molecular basis of Ca^{2+} sensitizing mechanisms in smooth muscles.

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